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PRINCIPAL INVESTIGATOR: Vimla Band, Ph.D.

CONTRACTING ORGANIZATION: New England Medical Center
Boston, Massachusetts 02111

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Boston, Massachusetts 02111

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13. ABSTRACT *(Maximum 200 words)*

The proposed studies are aimed to understand the biochemical mechanism of conversion of normal mammary epithelial cells (MECs) into tumor cells. Previously, we isolated a novel serine protease-like gene NES1 (Normal Epithelial Cell Specific-1) that is expressed in normal mammary epithelial cells but is down-regulated in most breast cancer cell lines. We now demonstrate that stable expression of NES1 in the NES1-negative MDA-MB-231 breast cancer cell line suppressed the oncogenicity as revealed by inhibition of the anchorage-independent growth and tumor formation in nude mice. Fluorescence in-situ hybridization localized the NES1 gene to chromosome 19q13.3, a region that contains genes for related proteases, including the prostate specific antigen, and is rearranged in human cancers. Similar to breast cancer cell lines, prostate cancer cell lines also lacked NES1 mRNA and protein expression. Finally, we have now expressed and purified NES1 protein to homogeneity and we present data showing the protease activity of NES1 against a universal serine protease substrate (casein resourfin) and against glandular and plasma kallikrein substrates. Interestingly, the NES1 protein also exhibits protease activity against insulin like growth factor binding protein-3 (IGFBP-3). Together, these studies are aimed towards understanding the tumor suppressor role for NES1 protein in breast and other carcinomas.

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FOREWORD

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Vimla Band 6/17/98

PI - Signature Date

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INTRODUCTION

Carcinomas, the malignant tumors arising from epithelial cells, constitute the majority of human cancers. In nearly all cases, the etiology of these cancers is unknown. Malignant transformation represents a complex multi-step process in which genetic changes and environmental factors including radiation, viruses, carcinogens and dietary components, are considered to play a role (1). To gain insights into biochemical pathways involved in epithelial cell oncogenesis, we and others have utilized in vitro models of epithelial cell transformation. In one such model, we exposed a normal mammary epithelial cell strain 76N to fractionated doses of γ-irradiation in vitro, similar to the therapeutic regimen used for cancer treatment, which resulted in its tumorigenic conversion (2). In order to isolate genes whose products contributed toward oncogenic transformation in this system, we carried out subtractive hybridization between normal parental cell strain 76N and its radiation-transformed cell line 76R-30 to identify differentially expressed mRNAs. This strategy resulted in the isolation of a novel putative serine protease, NES1 (normal epithelial specific-1), whose mRNA expression was observed in 76N cells but was down-regulated in 76R-30 cells (3). Using a panel of normal and tumor mammary epithelial cell lines, we showed that the expression of NES1 mRNA and protein was absent in a majority of breast tumor cell lines (3).

The predicted NES1 polypeptide showed high homology to a number of serine proteases (4), in particular the members of the trypsin family, the kallikrein family, and the family of proteases that activate the kringle domain-containing growth factors (5-7). The kallikrein family includes the prostate-specific antigen (PSA) which is increased in the serum of prostate cancer patients and serves as a prognostic marker (8,9). The kringle domain-containing growth factors include human tissue plasminogen activator and human hepatocyte growth factor activator which have been linked to oncogenesis (6,7,10,11). The former is increased during tumorigenic progression of cells, whereas the latter is a mitogenic growth factor for a known proto-oncogene, c-met (hepatocyte growth factor receptor) (12). The involvement of close homologues of NES1 in oncogenic transformation suggests a potential function for NES1 in cell growth.

Here, we show that transfection-mediated reconstitution of NES1 expression in a NES1-negative breast tumor cell line, MDA-MB-231, results in suppression of the tumorigenic phenotype both in vitro and in vivo. Using the fluorescent in situ hybridization (FISH) analysis, the NES1 gene was localized to chromosome 19q13.3 within the same region that contains PSA (9). Similar to mammary epithelial cells, NES1 mRNA and protein were expressed in normal and immortal prostatic epithelial cells but not in tumorigenic prostate epithelial cell lines. Together, these data suggest that NES1 plays a tumor suppressor role in breast, prostate, and possibly other epithelial cells.

PROPOSED SPECIFIC AIMS WERE

I. CHARACTERIZE THE EXPRESSION OF NES1 IN NORMAL AND TUMOR BREAST CELLS IN CULTURE AND IN TISSUE SPECIMENS.

1. Generate anti-NES1 antibodies.
2. Examine the expression of NES1 mRNA and protein in normal and tumor mammary cells in tissue sections and in cultures.
3. Examine the mechanisms of inducible NES1 expression.
4. Examine the effect of DNA damage on NES1 mRNA and protein expression.

II. ASSESS THE INFLUENCE OF ALTERATIONS IN NES1 EXPRESSION ON CELL GROWTH AND ONCOGENICITY.

1. Transfection of NES1 into mammary cells.
2. Growth properties and oncogenic behavior of NES1 transfectants.
3. Influence of reduced NES1 expression on growth, immortalization and oncogenicity of MECs.

III. CHARACTERIZE THE BIOCHEMICAL FUNCTIONS OF NES1.

1. Characterize the potential protease activity of NES1.
2. Examine non-protease biochemical functions of NES1 protein.
3. Mutational analysis of NES1 protein.

BODY OF THE REPORT

This section describes work accomplished from August 1997 to August 1998.

I. Expression of NES1 in a NES1-negative breast cancer cell line MDA-MB-231 results in reduced anchorage-independent growth and tumor formation in nude mouse (Aim II-2). The pattern of NES1 expression, with high levels in normal and immortal mammary epithelial cells, reduced levels in radiation-transformed 76R-30 cells and an essentially complete lack of expression in most mammary tumor cell lines (3), suggested a potential role of NES1 in tumor suppression. To directly assess if NES1 can function as a tumor suppressor protein, we introduced either the pCMVneo vector or NES1-pCMVneo plasmids into a breast cancer cell line, MDA-MB-231. The choice of MDA-MB-231 cell line was based on its lack of NES1 expression, its known ability to grow in an anchorage independent manner and its ability to form tumors when implanted in nude mouse (3,13). Following G418 selection, six independent stable clones each of the vector- and the NES1-transfected MDA-MB-231 cells were tested for the expression of NES1 mRNA and protein. As shown in Fig. 1A, 4 out of 6 NES1-transfected MDA-MB-231 clones (clone #s 2, 4, 5, and 6) expressed high levels of NES1 mRNA while the remaining two clones (clone #1, and 3) showed very little or no mRNA expression. When analyzed for NES1 protein, the four high mRNA-positive clones (clone # 2, 4, 5, and 6) showed considerably high levels of protein as compared to low mRNA-expressing clones (clone # 1, and

3) (Fig. 1B). As expected, none of the vector-transfectants showed any NES1 protein (lane 1 and data not shown). Based on the protein expression, the three strong positive clones (2, 4, and 5) were used for further analyses to examine the effect of NES1 on tumorigenicity.

Previous studies have demonstrated a direct correlation between the tumorigenic phenotype of cancer cell lines, such as MDA-MB-231, and the ability to grow in a anchorage-independent manner and to form tumors when implanted in nude mice (13). As shown in Fig. 2A, each of the three vector-transfected clones gave rise to colonies when seeded in soft agar, while the clonogenicity of the three NES1-transfectants was markedly decreased. The experiment was repeated three times, each in triplicate, with similar results. Thus, overexpression of NES1 in a NES1-negative breast cancer cell line abolished the ability of cells to grow in an anchorage-independent manner.

When $5x10^5$ cells of NES1-transfectant clone # 4 or a vector-transfectant were injected subcutaneously into the mammary gland area of mice, 5 out of 5 vector-transfected mice showed palpable tumors within 8-10 days of injection and these grew progressively reaching a 2.0 x 2.0 cm^2 size by 4 weeks (Fig. 2B). The tumor from one vector-transfectant implanted mouse was excised for histopathology and to assess the ability of tumor cells to grow in vitro. These experiments demonstrated that the tumor was an adenocarcinoma, as expected (13), and these cells proliferated in cell culture (data not shown). In contrast to vector-transfected MDA-MB-231 cells, none of the mice implanted with NES1-transfected MDA-MB-231 cells showed any palpable or visible tumors by 4 weeks. Mice were then sacrificed and the injected area was examined thoroughly for any non-palpable tumor growth. However, no tumor growth was observed. Taken together, these data clearly demonstrate the ability of NES1 to suppress the tumorigenicity in MDA-MB-231 cells.

Methods utilized:

1) Transfection. NES1 cDNA was cloned into the pCMV-neo vector using standard molecular biology procedures. 8 μg of HindIII linearized plasmid was used for transfection into MDA-MB-231 cells using the calcium phosphate co-precipitation method, as described earlier (14). After G418 (1 mg/ml) selection for 2 weeks, single colonies were isolated using cloning cylinders and subcultured at 1:3 split ratio.

2) Northern Blotting. Total cellular RNA was isolated from subconfluent monolayers of cells using the guanidinium-isothiocynate method. 10 μg samples of RNA were resolved on formaldehyde- 1.2% agarose gels and was transferred onto nylon membrane (Hybond-N; Amersham, Arlington Heights, IL). Hybridization was carried out with a ^{32}P-labeled NES1 cDNA probe (nucleotide 651 to 1072), as described earlier (3).

3) Western Blotting. Culture supernatants from vector- and NES1-transfected cells were collected following serum deprivation for 24 hours. 50 μg (breast cells) or 300 μg (prostate cells) of each supernatant (protein quantitation done using a Bicinchoninic acid protein assay reagent kit, Pierce Chem. Co., Rockford, IL) was separated on a 10% SDS-polyacrylamide gel (PAGE) and transferred to a polyvinylidene difluoride membrane (Immobilon-P Millipore, Marlborough, MA). The membranes were blocked with TBST (25 mM Tris, 0.15 M NaCl, 0.1 % Tween 20) containing 5% each of non-fat dry milk and BSA, incubated with either rabbit anti-NES1 antibody or monoclonal antibody against PSA (Ab-2, Neomarkers, Fremont, CA),

followed by either goat anti-rabbit IgG or goat anti-mouse IgG-horse-radish peroxidase conjugates, respectively. Enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

4) Anchorage-Independent Growth. MDA-MB-231 cells transfected with either the vector or NES1 were plated at 1 x 10^5/60 mm dish in a top layer of 0.3 % agar (Bacto Agar, DIFCO, Detroit, MI) with a bottom layer of 0.5% agar (both in 1 x complete MEM medium). Two ml of complete medium was added on top every 4th day and plates were examined for clonal growth under a microscope every other day and photographed after 2 weeks.

5) Tumor formation in Nude mice. Balb/C nude mice (4-6 weeks of age) were obtained from Jackson Laboratory (Bar Harbor, Maine) and allowed to adjust to the Institutional animal facility for 1 week before initiating the experiment. Subconfluent cultures of vector- or NES1-transfected MDA-MB-231 cells were released with trypsin/EDTA (0.025 % trypsin and 0.01 % EDTA) and resuspended in normal saline. 5x10^5 cells in a volume of 0.2 ml were injected subcutaneously into the mammary fat pad area (below the nipple). Five mice were injected per group. Mice were examined on alternate days for the presence of any palpable tumor growth, and tumor size was measured. Tumors were allowed to grow for 4 weeks at which time mice were photographed and sacrificed.

II. Localization of the NES1 gene to chromosome 19q13.3 by Fluorescence In Situ Hybridization analysis (New Aim). The potential role of NES1 as a tumor suppressor, as suggested by the above experiments, prompted us to examine the chromosomal localization of the NES1 gene. For this purpose, we performed pulsed-field gel electrophoresis of a Not1-digested bacterial artificial chromosome (Bac) clone and found that it contained a 140 Kb NES1 DNA insert. Southern blotting of an EcoRI-digest of DNA isolated from several single colonies of this Bac clone, compared with genomic DNA of 76N normal mammary epithelial cell strain using a NES1 cDNA probe indicated that the 140 Kb genomic clone contained nearly all of the NES1 gene (data not shown). This 140 Kb probe was used for FISH analysis. Thirty one metaphase spreads were analyzed for chromosomal localization of NES1. In all metaphase spreads, hybridization signals were found on both copies of the 19q (Fig. 3, as indicated by arrows). Longer metaphase spreads allowed localization of the NES1 gene to 19q13.3 and 90 % of these spreads had both chromatids stained. Propidium Iodide counter staining to evaluate chromosomal banding confirmed the presence of NES1 on 19q13.3 (data not shown). This analysis localizes NES1 gene to the same region where the gene for prostate cancer-associated serine protease PSA is localized (9). Furthermore, this region is known to undergo loss of heterozygosity in solid tumors including pancreatic carcinomas, astrocytoma, ovarian cancer and thyroid tumors (15), further supporting the potential tumor suppressor role of NES1.

Method:

Fluorescence In Situ Hybridization. Fluorescence in-situ hybridization (FISH) was performed using a commercial kit following the procedures recommended by the supplier (Oncor Inc. Gaithersburg, MD). A 140 kb human genomic DNA from bacterial artificial chromosome (Bac) library (obtained through Research Genetics Inc, Huntsville, AL., by screening the library with a NES1 cDNA probe) was used as a probe for FISH analysis. 300 ng of genomic NES1 DNA

probe was labeled with biotin-14-dCTP (Oncor, Inc) using the Bioprime DNA labeling system (Life Technologies, Grand Island, NY). The time and concentration of DNA were adjusted to produce biotinylated products of 100 to 500 bp in length as judged by agarose gel electrophoresis. The labeled DNA was purified through a microspin S-200 HR column (Pharmacia Biotechnologies, Piscataway, NJ) and precipitated with 3 M sodium acetate/ethanol in the presence of human COT-1 DNA (human placental DNA from GIBCO BRL, Grand Island, NY) and salmon sperm DNA in order to block non-specific hybridization due to any repetitive human sequences in the probe. The probe was resuspended in Hybrizol VI hybridization solution (Oncor, Inc). Prior to hybridization, the probe was denatured at 72°C for 10 min., preannealed for 30 min at 37°C, and chilled on ice.

Cytogenetic metaphase chromosome preparations were prepared from human peripheral blood lymphocytes stimulated with phytoheamagglutin (PHA) (15). Prior to denaturation, the slides were pretreated in 2X SSC (3 M NaCl, 0.3 M NaCitrate pH 7.0) for 30 minutes at 37°C followed by dehydration in an ethanol series (70-95%). Slides were denatured in 70% formamide, 2x SSC pH 7.0 at 70°C for 2 min, dehydrated in an ice-cold ethanol series (70-95%) and air dried. The denatured probe was added to the slide, covered with a glass coverslip, and sealed with rubber cement. The probe was hybridized in a moist chamber at 37°C overnight. Immunocytochemical detection of the hybridized probe was achieved using avidin-FITC and anti-avidin repetitive amplification steps (Oncor Inc.). The slides were mounted in antifade medium containing 4,6-diamino-2-phenylindole DAPI-II (Vysis Inc., Downers Grove, IL) for counterstaining of the chromosomes. Chromosomes were evaluated under a Zeiss fluorescence microscope equipped with appropriate filters for the visualization of FITC, DAPI and PI.

III) NES1 mRNA and protein expression is down-regulated in prostate cancer cell lines (New observation). The localization of NES1 gene to chromosome 19q13.3, where prostate specific antigen is localized, prompted us to analyze the status of NES1 expression in normal, immortal and tumor cells derived from the prostate gland. Northern blot analysis revealed that while NES1 was expressed at high levels in normal tissue from a prostactomy specimen, and two HPV-18 and SV40-immortalized prostate cell lines derived from normal prostate epithelial cells, four out of four prostate tumor cell lines completely lacked the NES1 mRNA expression (Fig. 4A). Importantly, the analysis of NES1 protein in supernatants revealed that the immortalized prostate cells secreted significant levels of NES1 protein whereas no protein was detected in the supernatants of tumorigenic prostate cell lines (Fig. 4B). Importantly, when these supernatants were tested for PSA levels, a significant levels of PSA protein was detected in one of the four (LNCaP) prostate cancer cell line. These data are consistent with the published reports on the secreted levels of PSA in LNCaP cells (16). Thus, similar to our previous results in breast cells, NES1 mRNA expression is downregulated during tumorigenic progression in prostate cancer. Taken together, these experiments further suggest that NES1 may play a tumor suppressor role in breast cancer, as well as in prostate cancer.

[The studies presented above are now in Press in Cancer Research, see appendix Ms. # 1]

IV) Further purification of NES1 protein (AIM III). In the last year's report, we presented partial purification of the NES1 protein using supernatants collected from NES1-expressing Sf21 cells using ammonium sulphate precipitation and separation on SP-Sepharose fast flow ion exchange column. We have now achieved a complete purification of NES1 protein by adding

a Gel filtration chromatography step to the previous protocol.

Method: Fractions containing NES1 from ion exchange column were pooled, concentrated and desalted using Centricon 10. Biogel 60 (Biorad, M.W. cutoff 60,000 kDa) was soaked in 10 mM Tris, 150 mM NaCl buffer overnight and packed in a 1.0 cm x 50 cm column at a flow rate 70 μl/min. The column was calibrated using Pharmacia's low molecular weight gel filtration standards. Ion exchange chromatography-purified and concentrated NES1 samples was loaded on Biogel column, run in the above buffer and O.D. 280 nm was monitored (Fig. 5A). 50 μl of fractions having O.D. 280 nm were run on 10% SDS PAGE and protein was visualized by immunoblotting (Fig. 5B). Fractions 1-9 were analyzed by silver staining (Fig. 5C). Note that fractions 6,7 and 8 showed only one band of 30 kDa corresponding to the NES1 protein.

V) Characterization of NES1 as a protease (Aim III). NES1 protein was purified from supernatants of baculovirus-infected Sf21 cells using ammonium sulfate fractionation and SP-sepharose fast flow ion-exchange chromatography (data presented in the last year's report) followed by gel-filtration chromatography (Fig. 5A). This yielded a homogeneously pure NES1 protein (assessed by silver staining and immunoblotting Fig. 5B and C). This material was used to test the protease activity of NES1. Purified NES1 was proteolytically active as measured by the release of resourfin-labeled peptides of Casein Resourfin (Boehringer Mannheim), a universal protease substrate into TCA soluble fractions (Fig. 6A). In addition, NES1 protein exhibited proteolytic activity similar to PSA against kallikrein-substrates (S-2266 and S-2302 purchased from DiaPharma Group Inc. West Chester, OH) as assessed by release of the paranitroaniline group (Fig. 6B and C). These initial analyses strongly indicate that NES1 is proteolytically active.

VI) Protease activity of NES1 against IGFBP3 (New Aim). As mentioned in the original grant, NES1 is highly homologous with serine proteases of the trypsin-like family, kallikrein family and activators of kringle family. The chromosomal localization of NES1 gene places NES1 in the kallikrein family of genes which are clustered on chromosome 19q13.3. This family includes a well characterized protein, human prostate specific antigen.

Recent studies have shown a role for kallikreins, such as PSA, in cleaving insulin-like growth factor-binding protein-3 (IGFBP-3), whereas IGFBP-1,-2,-4, and -6 were not cleaved significantly (17, 18). Based on these findings, we examined the potential protease activity of NES1 protein against IGFBP3 protein. As seen in Fig. 7 similar to PSA, NES1 protein cleaved IGFBP3 protein. These data show that like PSA NES1 can target the IGFBP3.

Method:

IGFBP proteolysis. Proteolytic digestion was carried out in PBS pH 7.4, with 0.5 mM $CaCl_2$ in a total volume of 40 μl. Approximately 30,000 cpm of ^{125}I-labeled IGFBP3 were used. Reaction was initiated by adding NES1 (100 μg/ml), or PSA (100 or 200 μg/ml). The digestion mixture was incubated at 37^{0}C for 6 hrs, and the reaction was terminated by adding PMSF (1 mM). The digestion mixture was then subjected to SDS-12.5% PAGE under non-reducing conditions at 60 V overnight. Gel was dried and autoradiographed.

Summary and Conclusions.

Our efforts to isolate genes that were differentially expressed in a normal mammary epithelial cell strain 76N and its γ-irradiation-transformed derivative, led to isolation of a novel serine protease whose expression was down-regulated in a majority of breast tumor cell lines. Introduction of NES1 into a NES1-negative breast tumor cell line MDA-MB-231 suppressed the tumorigenic phenotype of these cells, as revealed by an essentially complete suppression of anchorage-independent growth in soft agar and growth as implanted tumors in nude mice. The potential tumor suppressor role of NES1 is further supported and expanded by our studies using FISH analysis, which localized NES1 gene on chromosome 19 region q13.3 a region that is rearranged in a variety of human solid tumors. NES1 mRNA and protein expression were also observed in normal and immortal prostate epithelial cells with an essentially undetectable level of expression in prostate tumor cell lines. All of these independent lines of evidence strongly argue for a tumor suppressor role of NES1. Finally, purified NES1 exhibited protease activity against a universal substrate (Casein Resourfin) and plasma and glandular kallikreins. Based on studies that PSA cleaves insulin-like growth factor-binding protein-3 (IGFBP-3), we analyzed the protease activity of NES1 against IGFBP-3. Our preliminary data suggest that NES1 exhibits protease activity against IGFBP-3. IGFBP-3 has been shown to have IGF1-dependent and IGF1-independent inhibitory effects on cell growth (18). Interestingly, one of the cleavage product is a 16 kDa fragment which is known to be an potent inhibitor of IGF-induced cell growth (19). Therefore, loss of NES1 in tumor cells may influence this negative growth regulation by cells.

Altogether, our studies support a novel tumor suppressor function for the serine protease NES1 which is transcriptionally down-regulated during breast and prostate tumor progression. Given this information, it is likely that NES1 may also be involved in a critical aspect of regulating normal epithelial cell growth and/or differentiation.

A new specific aim proposed for study during the coming year.

-We will express NES1 gene into 76R-30 (cells that express dramatically low levels of NES1 mRNA), and a number of NES1-negative breast tumor cell lines and compare these for the expression of IGFI,IGFII, and IGFBP1-6 proteins.

-We will examine the proteolytic activity of NES1 against IGFBP1-6 as well as other specific substrates such as TGFβ.

These studies are proposed to provide further insights into the specific targets of NES1 protease that are potential candidates that may mediate its tumor suppressor function.

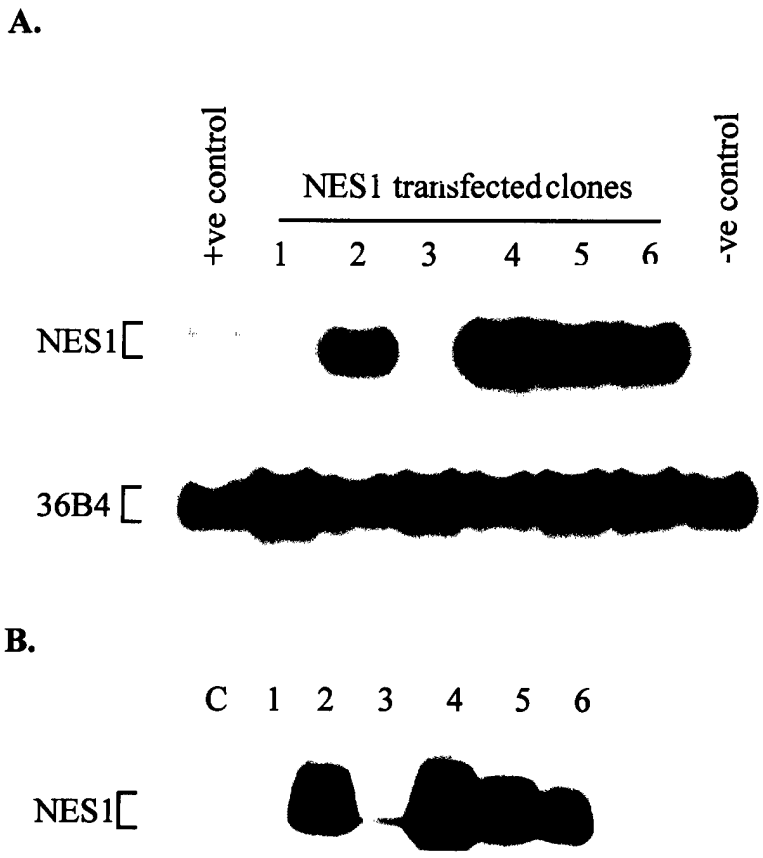


Fig. 1 A. Northern blot analysis of NES1 mRNA expression in NES1-transfected MDA-MB-231 cells. Nylon membranes with 10 μg of total RNA from different NES1-transfected clones (1-6) of MDA-MB-231 cells were hybridized with a ^{32}P-labelled NES1 probe and visualized by autoradiography. Positive (+ve) control, normal mammary epithelial 76N cells; Negative (-ve) control, vector-transfected MDA-MB-231 cells. 36B4 probe was used as a loading control.
B. Western blot analysis of NES1 protein in NES1-transfected MDA-MB-231 cells. Aliquots of culture supernatant derived from different NES1-transfected clones (1-6) of MDA-MB-231 cells containing 50 μg of protein were resolved by a SDS-10 % PAGE and transferred to PVDF membrane. Membranes were immunoblotted with an anti-NES1 antiserum followed by goat anti-rabbit IgG conjugated to horse-radish peroxidase. Detection was by enhanced chemiluminescence. Control (C), supernatant from vector-transfected MDA-MB-231 cells.

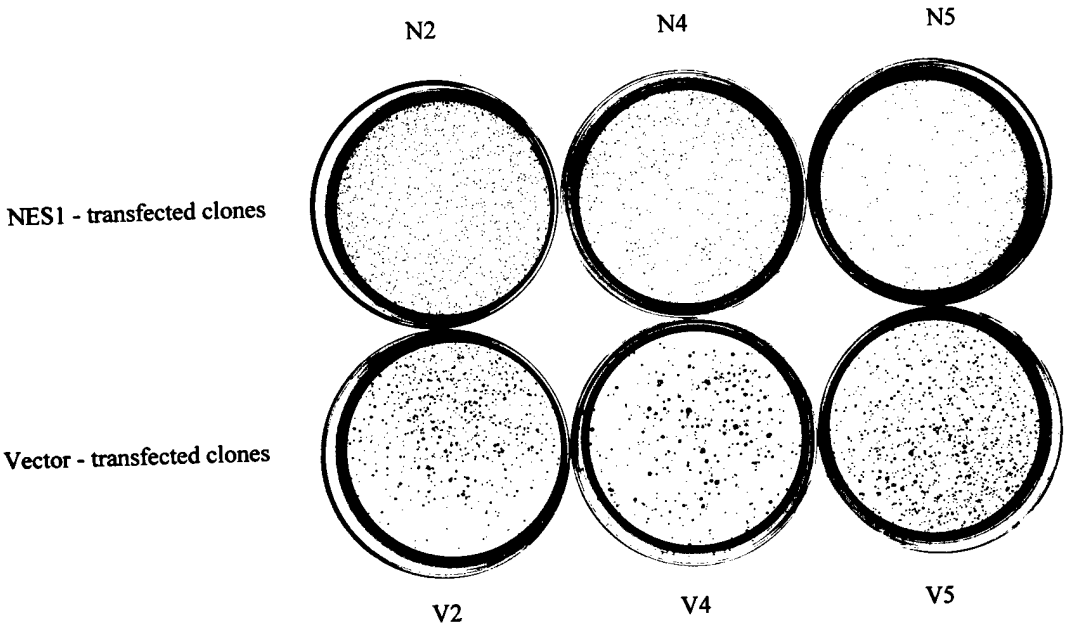


Fig. 2 A. Anchorage-independent growth of NES1-transfected clones. Various clones of vector-transfected (V2, V4, V5) or NES1-transfected (N2, N4, N5) MDA-MB-231 cells (1 X 10^5/60 mm dish) were plated in soft agar and colonies were photographed after 2 weeks.

NES1 - transfected cells

Vector - transfected cells

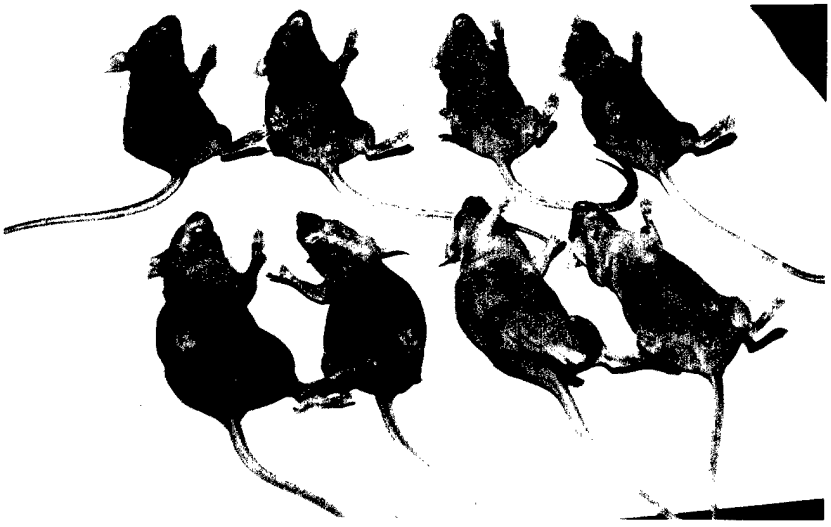


Fig. 2 B. Growth of vector- or NES1-transfected MDA-MB-231 cells as tumors upon implantation in nude mice. $5x10^5$ cells of vector or NES1-transfected MDA-MB-231 cells were injected subcutaneously into the mammary fat pad area below the nipple. Tumors were allowed to grow for 4 weeks at which time the mice were photographed and sacrificed.

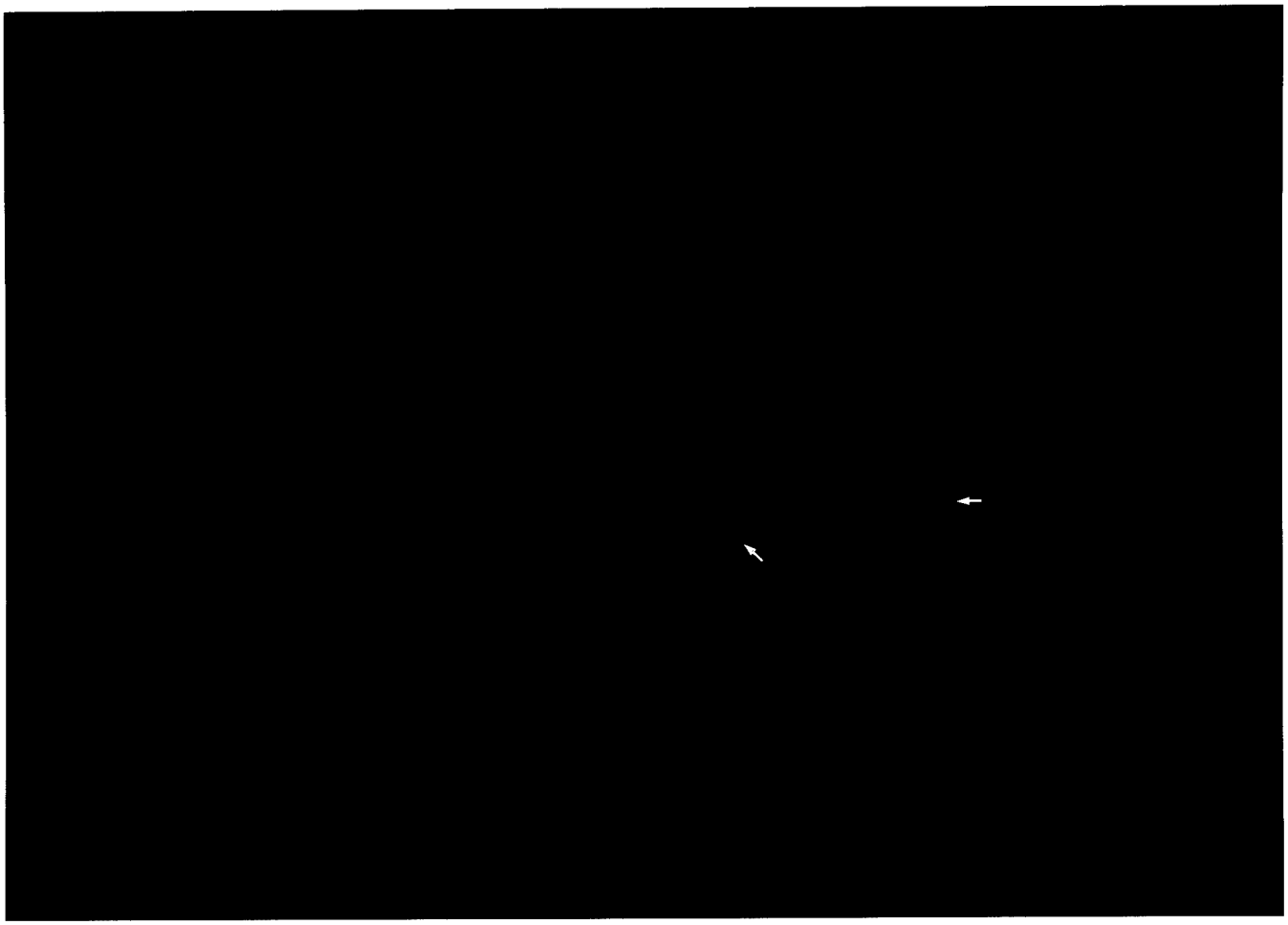


Fig. 3. Localization of NES1 gene by Fluorescence In Situ Hybridization (FISH) analysis. FISH analysis was performed using a commercial kit following the procedures recommended by the supplier (Oncor Inc.) using a biotin-14-dCTP labeled 140 kB NES1 genomic DNA probe. Cytogenetic metaphase chromosome preparations were from human peripheral blood lymphocytes stimulated with PHA. Immunocytochemical detection of the hybridized probe was achieved using avidin-FITC and anti-avidin repetitive amplification steps. The slides were mounted in antifade medium containing 4,6-diamino-2-phenylindole DAPI-II for counterstaining of the chromosomes. Chromosomes were evaluated under a Zeiss fluorescence microscope equipped with appropriate filters for the visualization of FITC and DAPI. Arrows point to NES1-specific hybridization signals in the telomeric region of the long arm of both chromosomes 19 at band q13.3.

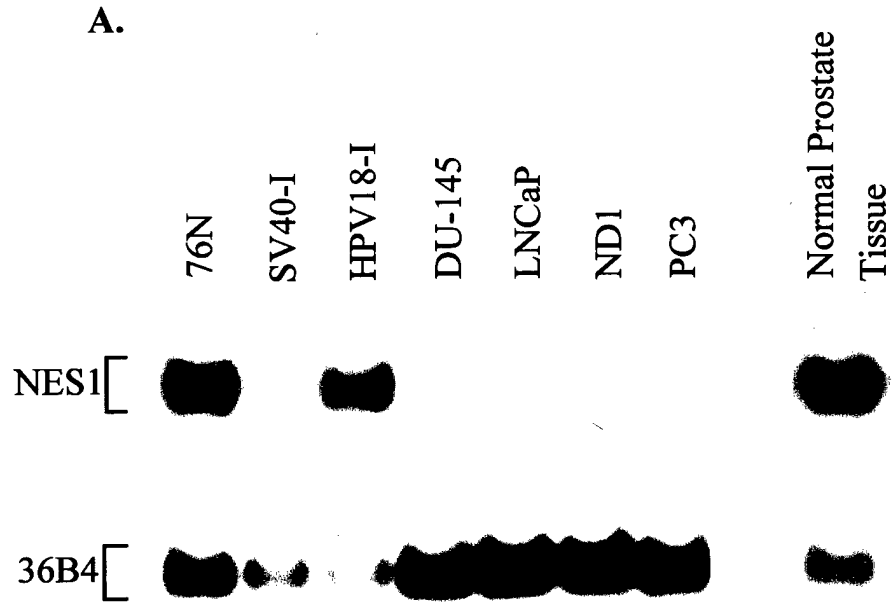


Fig. 4 A. Northern blot analysis of NES1 mRNA expression in prostate-derived normal, immortalized and tumor epithelial cells. Nylon membranes with 10 μg of total RNA from normal prostate tissue, immortalized prostate cell lines SV40-I and HPV18-I, and prostate tumor cell lines DU-145, LNCaP, ND1 and PC3, were hybridized with a ^{32}P-labelled NES1 probe and visualized by autoradiography. 76N normal mammary epithelial cell strain was used as a positive control. 36B4 probe was used as a loading control.

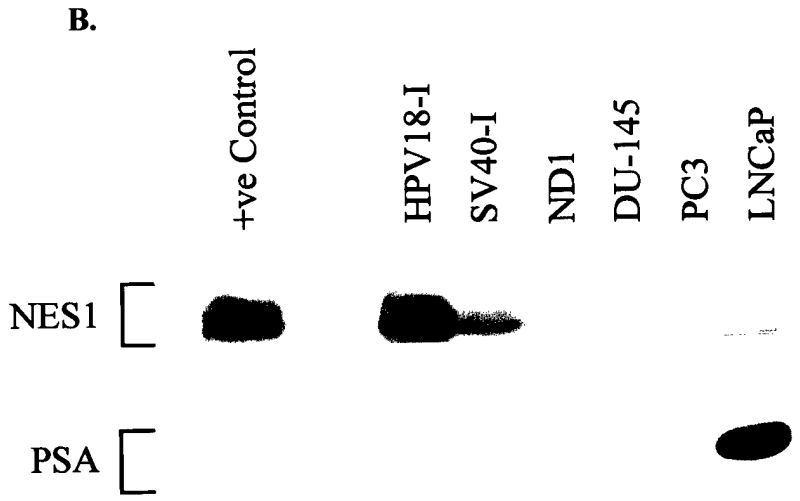
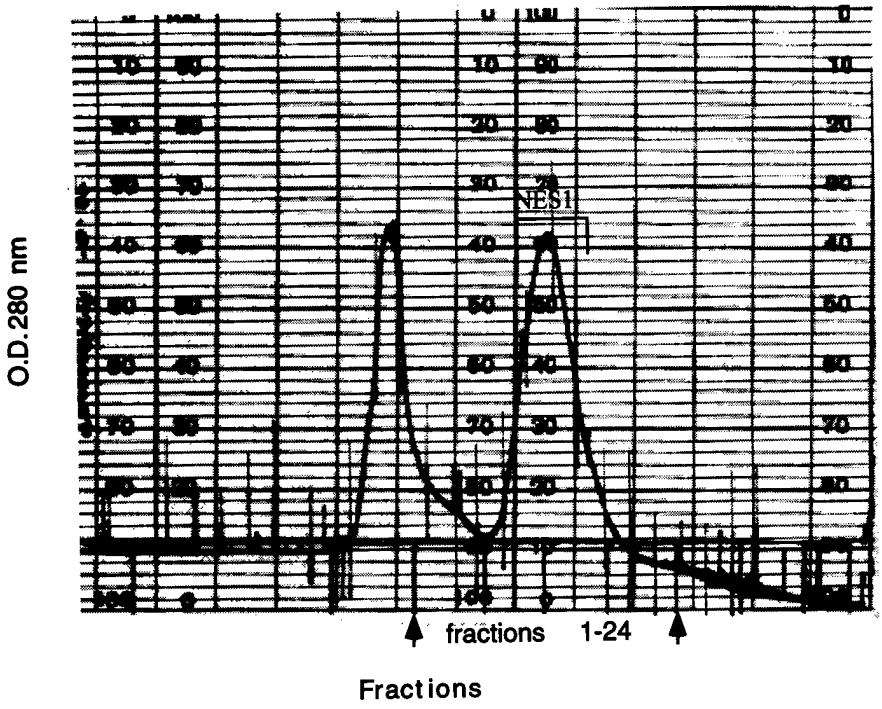


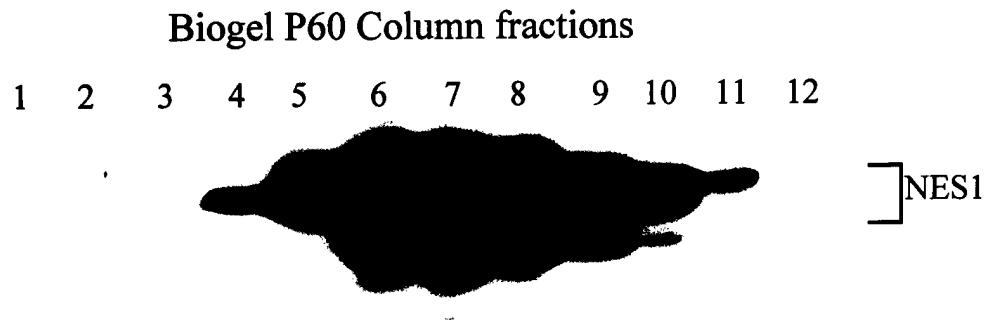
Fig. 4 B. Western blot analysis of NES1 protein in prostate-derived immortalized and tumor epithelial cell lines. Aliquots of culture supernatants derived from different cells (as indicated above) containing 300 μg protein were resolved by a SDS-10 % PAGE and transferred to PVDF membrane. Membranes were immunoblotted either with an anti-NES1 antiserum (above panel) or with an anti-PSA monoclonal antibody followed by goat anti-rabbit IgG or goat anti-mouse IgG horse-radish peroxidase conjugates, respectively. Detection was by enhanced chemiluminescence. Positive (+ve) control, supernatant from NES1-transfected MDA-MB-231 cells.



Gel filtration chromatogram
Biogel P60 column 1.0cmx50cm, flow rate 70ul/min
Chart speed 2cm/hour, Detector sensitivity 0.05

Fig. 5. (A) Gel filtration chromatography. Fractions containing NES1 from ion exchange column were pooled, concentrated and desalted using Centricon 10. Biogel 60 (Biorad, M.W. cutoff 60,000 kDa) was soaked in Tris 10 mM, NaCl 150 mM buffer overnight and packed in a 1.0 cm x 50 cm column, flow rate 70 μl/min. The column was calibrated using Pharmacia's LMW gel filtration standards. NES1 purified and concentrated from ion exchange chromatography was loaded on Biogel column, run in the same buffer and O.D. 280 nm was monitored.

B



C

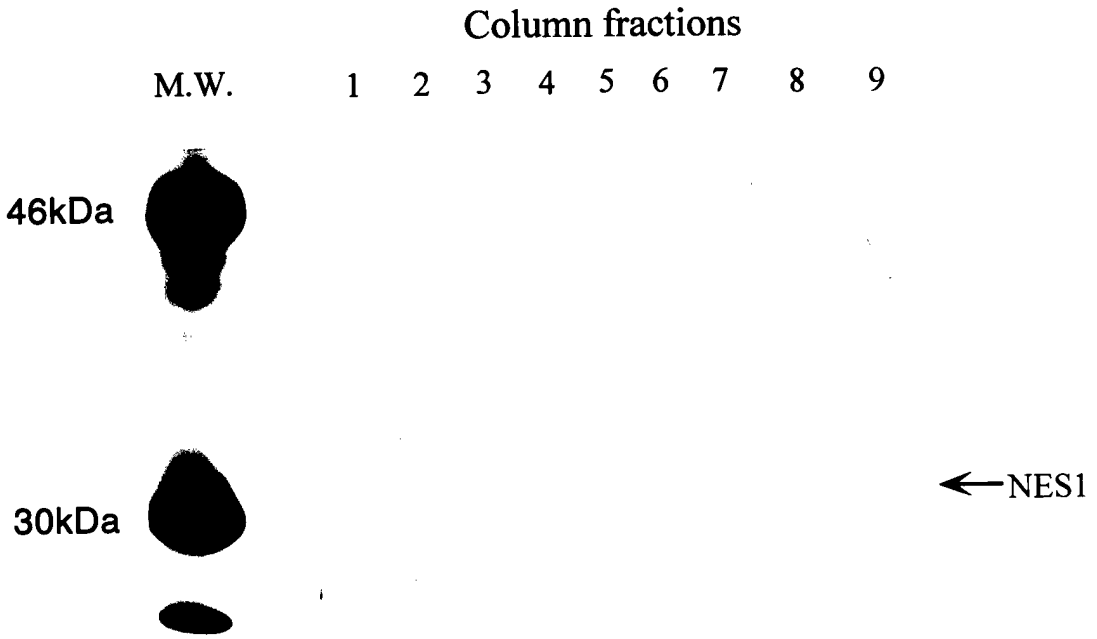
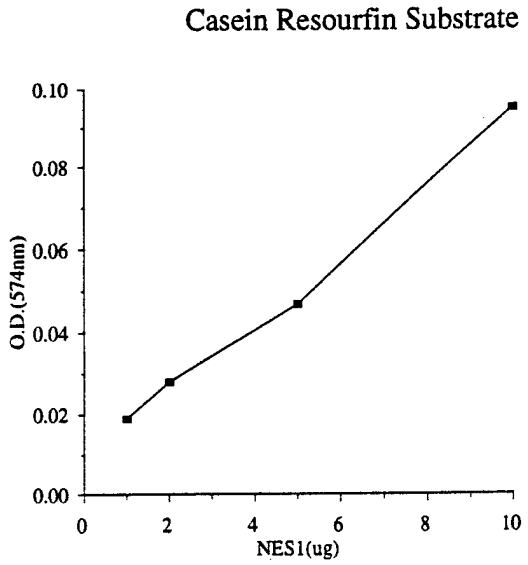
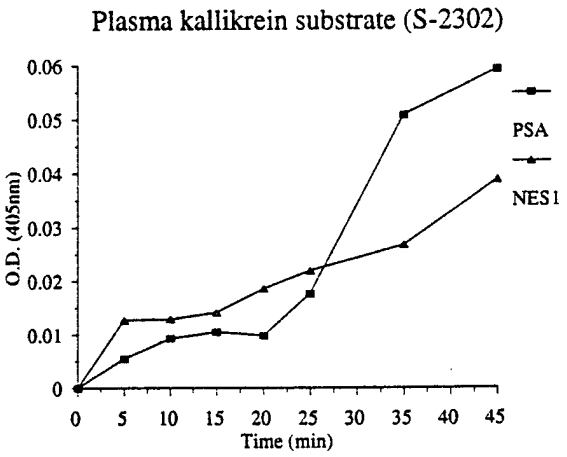


Fig. 5. (B) 50 μl of fractions having O.D. 280 nm were run on SDS-10.5% PAGE, and transferred to PVDF membrane. Membranes were immunoblotted with an anti-NES1 antiserum followed by goat anti-rabbit IgG conjugated to horse-radish peroxidase. Detection was by enhanced chemiluminescence (C). 50 μl of fractions having O.D. 280 nm were run on SDS-10.5% PAGE, and protein was visualized with the help of silver staining. Note that fractions 6,7 and 8 showed only one band of 30 kDa corresponding to the NES1 protein.

A.



B.



C.

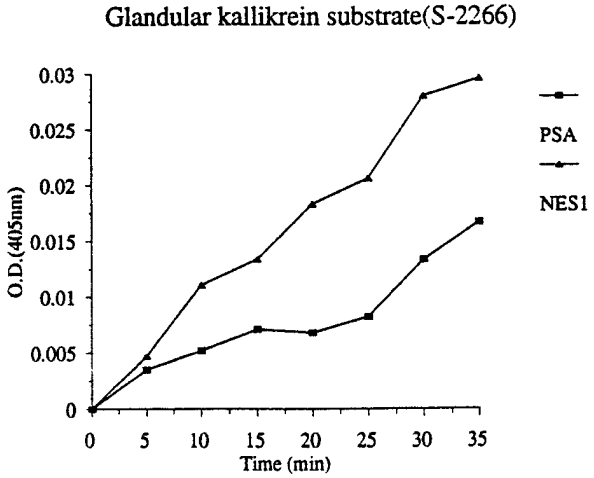


Fig. 6 A. Increasing concentration of NES1 (1-10ug) in 0.2 M Tris-HCl, pH 7.8, $CaCl_2$ 0.02 M was incubated with 50 ul of 0.4% Casein Resourfin substrate at 37 ^{0} C overnight. 5% TCA was then added to stop the reaction incubated further at 37^{0}C for 10 min. centrifuged and 400 ul supernatant was mixed with 600 ul assay buffer (0.5M Tris-HCl, pH 8.8). Absorbance at 574 nm was read immediately against the blank. **6 B and C.** Hydrolysis of synthetic peptide substrates (S2266 and S2302) was measured at 405 nm at 37^{0}C in a Beckman DU-70 spectrophotometer. Substrate 1x10^{-3}mol/L was taken in 1 ml of 0.05 mol/L Tris-HCl buffer,pH 8.4. Reaction was initiated by adding 10 ug of NES1 or PSA (positive control). Increase in absorbance was recorded and plotted against time.

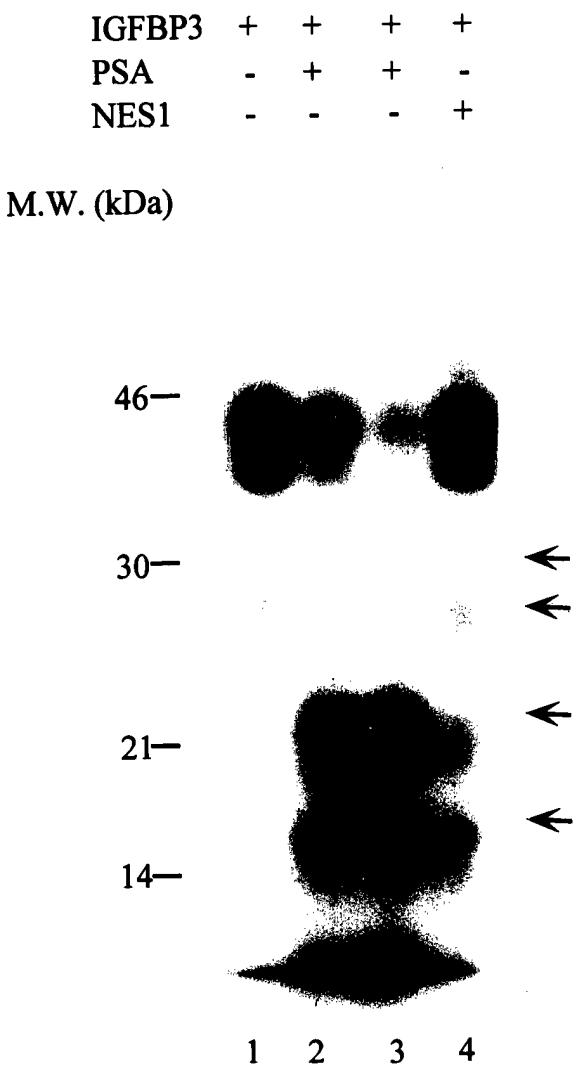


Fig. 7. IGFBP3 Proteolytic digestion was carried out in PBS pH 7.4, with 0.5 mM $CaCl_2$ in a total volume of 40 μl using 30,000 cpm of ^{125}I-labeled IGFBP3. Reaction was initiated by adding NES1 (100 μg/ml), or PSA (100 or 200 μg/ml, lanes 2 and 3 respectively). The digestion mixture was incubated at 37^{0}C for 6 hrs, and the reaction was terminated by adding PMSF (1 mM). The digestion mixture was then subjected to SDS-12.5% PAGE under non-reducing conditions. Gel was dried and autoradiographed. Arrows indicate proteolytic products of IGFBP3.

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The Role for NES1 Serine Protease as a Novel Tumor Suppressor[1]

Jaya Goyal, Karen M. Smith, Janet M. Cowan, David E. Wazer, Sam W. Lee, and Vimla Band[2]

Department of Radiation Oncology [J.G., D.E.W., V.B.], and Pathology [J.M.C.], New England Medical Center, and Department of Biochemistry [V.B], and Genetics Program [K.M.S., V.B.], Tufts University School of Medicine, Boston, Massachusetts 02111 and Department of Gerontology (S.W.L), Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115.

[1]This work was supported by Department of Defense Breast Cancer Program

[2]To whom requests for reprints should be addressed, at Department of Radiation Oncology, Box # 824, New England Medical Center, 750 Washington Street, Boston, MA 02111. Phone: (617)-636-4776; Fax: (617)-636-6205; E.Mail: VBAND@opal.Tufts.Edu.

Running Title: NES1 as a tumor suppressor

Key Words: tumor suppressor, breast cancer, serine protease

Abstract

Previously, we isolated a novel serine protease-like gene NES1 (Normal Epithelial Cell Specific-1) that is expressed in normal mammary epithelial cells but is down-regulated in most breast cancer cell lines. Here, we demonstrate that stable expression of NES1 in the NES1-negative MDA-MB-231 breast cancer cell line suppressed the oncogenicity as revealed by inhibition of the anchorage-independent growth and tumor formation in nude mice. Fluorescence in-situ hybridization localized the NES1 gene to chromosome 19q13.3, a region that contains genes for related proteases including the prostate specific antigen, and is rearranged in human cancers. Similar to breast cancer cell lines, prostate cancer cell lines also lacked NES1 mRNA and protein expression. Together, these results strongly suggest a tumor suppressor role for NES1 in breast and prostate cancer.

Introduction

Carcinomas, the malignant tumors arising from epithelial cells, constitute the majority of human cancers. In nearly all cases, the etiology of these cancers is unknown. Malignant transformation represents a complex multi-step process in which genetic changes and environmental factors including radiation, viruses, carcinogens and dietary components, are considered to play a role (1).

To gain insights into biochemical pathways involved in epithelial cell oncogenesis, we and others have utilized in vitro models of epithelial cell transformation. In one such model, we exposed a normal mammary epithelial cell strain 76N to fractionated doses of γ-irradiation in vitro, similar to therapeutic regimen used for cancer treatment, which resulted in its tumorigenic conversion (2). In order to isolate genes whose products contributed toward oncogenic transformation in this system, we carried out subtractive hybridization between normal parental cell strain 76N and its radiation-transformed cell line 76R-30 to identify differentially expressed mRNAs. This strategy resulted in the isolation of a novel putative serine protease, NES1 (normal epithelial specific-1), whose mRNA expression was observed in 76N cells but was down-regulated in 76R-30 cells (2). Using a panel of normal and tumor mammary epithelial cell lines, we showed that the expression of NES1 mRNA and protein was absent in a majority of breast tumor cell lines (2).

The predicted NES1 polypeptide showed high homology to a number of serine proteases (3), in particular the members of the trypsin family, the kallikrein family, and the family of proteases that activate the kringle domain-containing growth factors (4-6). The kallikrein family includes the prostate-specific antigen (PSA) which is increased in the serum of prostate cancer

patients and serves as a prognostic marker (7, 8). The kringle domain-containing growth factors include human tissue plasminogen activator and human hepatocyte growth factor activator which have been linked to oncogenesis (5, 6, 9, 10). The former is increased during tumorigenic progression of cells, whereas the latter is a mitogenic growth factor for a known proto-oncogene, c-met (hepatocyte growth factor receptor) (11). The involvement of close homologues of NES1 in oncogenic transformation suggests a potential function for NES1 in cell growth.

Here, we show that transfection-mediated reconstitution of NES1 expression in a NES1-negative breast tumor cell line, MDA-MB-231, results in suppression of the tumorigenic phenotype both in vitro and in vivo. The NES1 gene localized to chromosome 19q13.3 within the same region that contains PSA (8). Similar to mammary epithelial cells, NES1 mRNA and protein was expressed in normal and immortal prostatic epithelial cells but not in tumorigenic prostate epithelial cell lines. Together, these data suggest that NES1 plays a tumor suppressor role in breast, prostate, and possibly other epithelial cells.

Materials and Methods

Tissue sample and Cell lines. Normal prostate tissue was from a prostactomy specimen obtained from the Beth Israel Deaconess Medical Center. Normal prostate epithelial cells immortalized with the human papilloma virus type 18 (designated as HPV18-I cells) or simian virus 40 large T antigen (SV40-I) (obtained from Dr. J. S. Rhim) (12, 13), the ND1 prostate cancer cell line (obtained from Dr. P. Narayan) (14), and the MDA-MB-231 breast cancer cell line (obtained from Dr. Ruth Lupu) have been described (15). PC3, DU-145, and LNCaP prostate cancer cell lines were obtained from American Tissue Type Collection. All cell lines

used in this study were grown in α-minimum essential medium with 10 % fetal calf serum (16).

Transfection. NES1 cDNA was cloned into pCMV-neo vector and 8 μg of HindIII linearized plasmid was used for transfection into MDA-MB-231 cells using the calcium phosphate co-precipitation method as described earlier (16). After G418 (1 mg/ml) selection for 2 weeks single colonies were isolated and subcultured at 1:3 split ratio.

Northern Blotting. Total cellular RNA was isolated from subconfluent monolayer cells using the guanidinium-isothiocynate method. 10 μg of each RNA was resolved on formaldehyde-1.2% agarose gel and was transferred onto a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL). Hybridization was carried out with ^{32}P-labeled NES1 cDNA probe (nucleotide 651 to 1072) as described earlier (2).

Western Blotting. Culture supernatants from vector- and NES1-transfected cells were collected for 24 hours of prior serum deprivation. 50 μg (breast cells) or 300 μg (prostate cells) of each supernatant (protein quantitation done using a Bicinchoninic acid protein assay reagent kit, Pierce Chem. Co., Rockford, IL) was separated on a 10% SDS-polyacrylamide gel (PAGE) and transferred to a polyvinylidene difluoride membrane (Immobilon-P Millipore, Marlborough, MA). The membranes were blocked with TBST (25 mM Tris, 0.15 M NaCl, 0.1 % Tween 20) containing 5% each of non-fat dry milk and BSA, incubated with either rabbit anti-NES1 antibody or monoclonal antibody against PSA (Ab-2, Neomarkers, Fremont, CA), followed by either goat anti-rabbit IgG or goat anti-mouse IgG-horse-radish peroxidase conjugates, respectively. Enhanced chemiluminescence detection was performed according to manufacturers' instructions (Amersham, Arlington Heights, IL).

Anchorage-Independent Growth. MDA-MB-231 cells transfected with either vector

or NES1 were plated at 1x 10^5/60 mm dish in a top layer of 0.3 % agar (Bacto Agar, DIFCO, Detroit, MI) with a bottom layer of 0.5% agar (both in 1 X complete MEM medium). Two ml of complete medium was added on top every 4^{th} day and plates were examined for clonal growth under a microscope every other day and photographed after 2 weeks.

Tumor formation in Nude mice. Balb/C nude mice (4-6 weeks of age) were obtained from Jackson Laboratory (Bar Harbor, Maine) and allowed to adjust to the Institutional animal facility for 1 week before initiating the experiment. Subconfluent cultures of vector or NES1-transfected MDA-MB-231 cells were released with trypsin/EDTA (0.025 % trypsin and 0.01 % EDTA) and resuspended in normal saline. $5x10^5$ cells in a volume of 0.2 ml were injected subcutaneously into the mammary fat pad area (below the nipple). Five mice were injected per group. Mice were examined on alternate days for the presence of any palpable tumor growth, and tumor size was measured. Tumors were allowed to grow for 4 weeks at which time mice were photographed and sacrificed.

Fluorescence In Situ Hybridization. Fluorescence in-situ hybridization (FISH) was performed using a commercial kit following the procedures recommended by the supplier (Oncor Inc. Gaithersburg, MD). A 140 kb human genomic DNA from bacterial artificial chromosome (Bac) library (obtained through Research Genetics Inc, Huntsville, AL., by screening the library with a NES1 cDNA probe) was used as a probe for FISH analysis. 300 ng of genomic NES1 DNA probe was labeled with biotin-14-dCTP (Oncor, Inc) using the Bioprime DNA labeling system (Life Technologies, Grand Island, NY). The time and concentration of DNA were adjusted to produce biotinylated products of 100 to 500 bp in length as judged by agarose gel electrophoresis. The labeled DNA was purified through a microspin S-200 HR column

(Pharmacia Biotechnologies, Piscataway, NJ) and precipitated with 3 M sodium acetate/ethanol in the presence of human COT-1 DNA (human placental DNA from GIBCO BRL, Grand Island, NY) and salmon sperm DNA in order to block non-specific hybridization due to any repetitive human sequences in the probe. The probe was resuspended in Hybrizol VI hybridization solution (Oncor, Inc). Prior to hybridization, the probe was denatured at 72°C for 10 min., preannealed for 30 min at 37°C, and chilled on ice.

Cytogenetic metaphase chromosome preparations were prepared from human peripheral blood lymphocytes stimulated with phytoheamagglutin (PHA) (17). Prior to denaturation, the slides were pretreated in 2X SSC (3 M NaCl, 0.3 M NaCitrate pH 7.0) for 30 minutes at 37°C followed by dehydration in an ethanol series (70-95%). Slides were denatured in 70% formamide, 2x SSC pH 7.0 at 70°C for 2 min, dehydrated in an ice-cold ethanol series (70-95%) and air dried. The denatured probe was added to the slide, covered with a glass coverslip, and sealed with rubber cement. The probe was hybridized in a moist chamber at 37°C overnight. Immunocytochemical detection of the hybridized probe was achieved using avidin-FITC and anti-avidin repetitive amplification steps (Oncor Inc.). The slides were mounted in antifade medium containing 4,6-diamino-2-phenylindole DAPI-II (Vysis Inc., Downers Grove, IL) for counterstaining of the chromosomes. Chromosomes were evaluated under a Zeiss fluorescence microscope equipped with appropriate filters for the visualization of FITC, DAPI and PI.

Results

NES1-transfected cells lack anchorage independence as well as ability to grow in nude mouse. The pattern of NES1 expression, with high levels in normal cells, reduced levels

in radiation-transformed 76R-30 and an essentially complete lack of expression in most mammary tumor cell lines (2) suggested a potential role of NES1 in tumor suppression. To directly assess if NES1 can function as a tumor suppressor protein, we introduced either pCMVneo vector or NES1-pCMVneo plasmids into a breast cancer cell line, MDA-MB-231. The choice of MDA-MB-231 cell line was based on its lack of NES1 expression and its known ability to grow in an anchorage independent manner as well as its ability to form tumors when implanted in nude mouse (2, 15). Following G418 selection, six independent stable clones each of the vector and the NES1-transfected MDA-MB-231 cells were tested for the expression of NES1 mRNA and protein. As shown in Fig. 1A, 4 (clone #s 2, 4, 5, and 6) out of 6 NES1-transfected MDA-MB-231 clones expressed high levels of NES1 mRNA while the remaining two (clone #1, and 3) showed very little or no mRNA expression. When analyzed for NES1 protein secretion four clones (clone # 2, 4, 5, and 6) showed considerably high levels of protein as compared to two other clones (clone # 1, and 3) (Fig. 1B). As expected none of the vector-transfectants showed any NES1 protein (lane 1 and data not shown). Based on the protein expression, the three strong positive clones (2, 4, and 5) were used for further analyses to examine the effect of NES1 on tumorigenicity.

Previous studies have demonstrated a direct correlation between the tumorigenic phenotype of cancer cell lines such as MDA-MB-231 and the ability to grow in a anchorage-independent manner and to form tumors when implanted into nude mice (15). As shown in Fig. 2A, all of the three vector-transfected clones formed colonies when grown in soft agar, while the clonogenicity of the three NES1-transfectants was markedly decreased. The experiment was repeated three times, each in triplicate, with similar results. Thus, overexpression of NES1 in

a NES1-negative breast cancer cell line abolished the ability of cells to grow in an anchorage-independent manner.

When $5x10^5$ cells of NES1-transfectant clone # 4 or a vector-transfectant were injected subcutaneously into the mammary gland area of mice, 5 out of 5 vector-transfected mice showed palpable tumors within 8-10 days of injection and these grew progressively reaching a 2.0 x 2.0 cm^2 size by 4 weeks (Fig. 2B). The tumor from one vector-transfectact implanted mouse was excised for histopathology and to assess the ability of tumor cells to grow in vitro. These experiments demonstrated that the tumor was an adenocarcinoma, as expected (15), and these cells proliferated in cell culture (data not shown). In contrast to vector-transfected MDA-MB-231 cells, none of the mice implanted with NES1-transfected MDA-MB-231 showed any palpable or visible tumors by 4 weeks. Mice were then sacrificed and the injected area was examined thoroughly for any non-palpable tumor growth. However, no tumor growth was observed. Taken together, these data clearly demonstrate the ability of NES1 to suppress tumorigenesis in MDA-MB-231 cells.

Localization of the NES1 gene to chromosome 19q13.3 by Fluorescence In Situ Hybridization analysis. The potential role of NES1 as a tumor suppressor suggested by the above experiments prompted us to examine the chromosomal localization of the NES1 gene. For this purpose, we performed pulsed-field gel electrophoresis of Not1-digested Bac clone and found that it contained a 140 Kb NES1 DNA insert. Southern blotting of an EcoRI-digest of DNA isolated from several single colonies of the Bac clone compared with genomic DNA of 76N normal mammary epithelial cell strain using a NES1 cDNA probe indicated that the 140 Kb genomic clone contained a nearly all of the NES1 gene (data not shown). This 140 Kb probe

was used for FISH analysis. Thirty one metaphase spreads were analyzed for chromosomal localization of NES1. In all metaphase spreads, hybridization signals were found on both copies of the 19q (Fig. 3, as indicated by arrows). Longer metaphase spreads allowed localization of the NES1 gene to 19q13.3 and 90 % of these spreads had both chromatids stained. Propidium Iodide counter staining to evaluate chromosomal banding confirmed the presence of NES1 on 19q13.3 (data not shown). This analysis localizes NES1 gene to the same region where the gene for prostate cancer-associated serine protease PSA is localized (8). Furthermore, this region is known to undergo loss of heterozygosity in solid tumors including pancreatic carcinomas, astrocytoma, ovarian cancer and thyroid tumors (17), consistent with a potential tumor suppressor role of NES1.

NES1 mRNA and protein expression is down-regulated in prostate cancer cell lines. The localization of NES1 gene to chromosome 19q13.3, where prostate specific antigen is localized, prompted us to analyze the status of NES1 expression in normal, immortal and tumor cells derived from the prostate gland. Northern blot analysis revealed that while NES1 was expressed at high levels in normal tissue from a prostactomy specimen, and two HPV-18 and SV40-immortalized normal prostate cell lines, four out of four prostate tumor cell lines completely lacked the NES1 mRNA expression (Fig. 4A). Importantly, the analysis of NES1 protein in the supernatants of the immortalized prostate cells secreted significant levels of NES1 protein whereas no protein was detected in the supernatants of prostate tumor cell lines (Fig. 4B). Importantly, when these supernatants were tested for PSA levels, a significant levels of PSA protein was detected in one of the four (LNCaP) prostate cancer cell line. These data are consistent with the published reports on the secreted levels of PSA in LNCaP cells (13). Thus,

similar to our previous results in breast cells, NES1 mRNA expression is downregulated during tumorigenic progression in prostate cancer. Taken together, these experiments suggest that NES1 may play a tumor suppressor role in breast as well as prostate cancer.

Discussion

Our efforts to isolate genes that were differentially expressed in a normal mammary epithelial cell line 76N and its γ-irradiation-transformed derivative, led to isolation of a novel serine protease whose expression was down-regulated in a majority of breast tumor cell lines (2). Here, we demonstrate that, similar to breast cancers, NES1 expression is also down-regulated at the mRNA level in prostate cancer-derived cell lines whereas both the mRNA and NES1 protein are abundantly expressed in immortal prostate epithelial cell lines. Together, the down-regulation of NES1 expression during tumorigenic progression in two different epithelial tumors further supports the likelihood that NES1 functions in regulating cell proliferation, differentiation or other traits that are deregulated during oncogenesis.

Based on its lack of expression in breast cancer cells, we examined the consequence of NES1 transfection into a NES1-negative breast tumor cell line MDA-MB-231, which allowed an assessment of several aspects of tumorigenic phenotype (2). We demonstrate that NES1 gene indeed suppressed the tumorigenic phenotype of these cells, as revealed by an essentially complete suppression of anchorage-independent growth in soft agar and growth as implanted tumors in nude mice. This effect was not due to an overall suppression of cell proliferation, as vector-transfected cells and NES1-transfected cells grow comparably under regular culture conditions.

The potential tumor suppressor role of NES1 is further supported and expanded by our studies using FISH analysis, which localized NES1 gene on chromosome 19 region q13.3. As we have reported earlier, NES1 is highly homologous with serine proteases of the trypsin-like family, kallikrein family and activators of kringle family. The chromosomal localization of NES1 gene places NES1 in the kallikrein family of genes which are clustered on chromosome 19q13.3. These results suggest that NES1 may be derived by gene duplication from a common ancestor of the kallikrein family of serine proteases. In humans, this family includes four members: human renal/pancreatic kallikrein, human glandular kallikrein, human prostate specific antigen, and the recently isolated protease M (18). Notably, except for protease M all other members of this family are secreted proteins (2, 3, 18). Interestingly, similar to NES1, protease M also exhibits a reduced expression in breast and prostate tumor cell lines compared to normal cells (18). While PSA levels are elevated in the serum of prostate cancer patients and their high levels represent a marker of poorer prognosis (7, 8), PSA was reported as marker of better prognosis in breast cancer (19). Thus, two additional members of the kallikrein family that co-localize on 19q13 with NES1 also appear to be closely related to the process of human oncogenesis. Finally, 19q13 is known to be rearranged in a variety of human solid tumors including pancreatic carcinomas, astrocytoma, ovarian cancer and thyroid tumors (17). Although any direct involvement of NES1 or other kallikrein family member in these rearrangements remains to be analyzed, the chromosomal location of NES1 is fully consistent with its role as a tumor suppressor.

The mechanism of how NES1 induces suppression of the tumorigenic phenotype is currently unknown. Given that NES1 is a secreted serine protease, it is likely that its targets

are also extracellular, either components of the extracellular matrix, extracellular growth regulatory molecules or cell surface receptors. It is well documented that normal cellular behavior is regulated by both positive and negative factors. NES1 could mediate its tumor suppressor role either by generating an inhibitory factor(s) or by terminating the action of an activating factor(s).

Recent studies have shown a role for kallikreins, such as PSA in cleaving insulin-like growth factor-binding protein-3 (IGFBP-3), whereas IGFBP-1,-2,-4, and -6 were not cleaved significantly (as reviewed in reference 20). IGFBP-3 has been shown to have IGF1-dependent and IGF1-independent inhibitory effects on cell growth and potential cleavage of IGFBP-3 by PSA may be expected to enhance cell growth, consistent with the correlation of the high levels of PSA and low levels of IGFBP-3 with tumor burden in prostate cancer (20, 21). However, a similar mechanism for NES1 would not account for its tumor suppressor function. Interestingly, whereas a decrease in the levels of IGFBP-3 was observed in the sera of patients with prostate cancer, a significant elevation of IGFBP-2 was noticed (22). Furthermore, patients with high serum levels of PSA also showed elevated levels of IGFBP-2 (22). Interestingly, breast carcinoma cells synthesize a number of IGFBPs, with ER-positive and negative cells secreting different types of IGFBPs (23). Recently, it has been shown that IGFBP-3 predisposes breast cancer cells to program cell death in a non-IGF-dependent manner (24). It is therefore possible that NES1 may target one or more IGFBP family members. Further studies will need to address if this is the case and if other targets of NES1 relevant to its tumor suppressor function exist.

Altogether, our studies support a novel tumor suppressor function for the serine protease

NES1 which is transcriptionally downregulated during breast and prostate tumor progression. Given this information, it is likely that NES1 may also be involved in a critical aspect of regulating normal epithelial cell growth and/or differentiation.

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Figure legends

Fig. 1 A. Northern blot analysis of NES1 mRNA expression in NES1-transfected MDA-MB-231 cells. Nylon membranes with 10 μg of total RNA from different NES1-transfected clones (1-6) of MDA-MB-231 cells were hybridized with a ^{32}P-labelled NES1 probe and visualized by autoradiography. Positive (+ve) control, normal mammary epithelial 76N cells; Negative (-ve) control, vector-transfected MDA-MB-231 cells. 36B4 probe was used as a loading control.

Fig. 1 B. Western blot analysis of NES1 protein in NES1-transfected MDA-MB-231 cells. Aliquots of culture supernatant derived from different NES1-transfected clones (1-6) of MDA-MB-231 cells containing 50 μg of protein were resolved by a SDS-10 % PAGE and transferred to PVDF membrane. Membranes were immunoblotted with an anti-NES1 antiserum followed by goat anti-rabbit IgG conjugated to horse-radish peroxidase. Detection was by enhanced chemiluminescence. Control (C), supernatant from vector-transfected MDA-MB-231 cells.

Fig. 2 A. Anchorage-independent growth of NES1-transfected clones. Various clones of vector-transfected (V2, V4, V5) or NES1-transfected (N2, N4, N5) MDA-MB-231 cells (1 X 10^5/60 mm dish) were plated in soft agar and colonies were photographed after 2 weeks.

Fig. 2 B. Growth of vector- or NES1-transfected MDA-MB-231 cells as tumors upon implantation in nude mice. $5x10^5$ cells of vector or NES1-transfected MDA-MB-231 cells were injected subcutaneously into the mammary fat pad area below the nipple. Tumors were allowed

to grow for 4 weeks at which time the mice were photographed and sacrificed.

Fig. 3. Localization of NES1 gene by Fluorescence In Situ Hybridization (FISH) analysis. FISH analysis was performed using a commercial kit following the procedures recommended by the supplier (Oncor Inc.) using a biotin-14-dCTP labeled 140 kB NES1 genomic DNA probe. Cytogenetic metaphase chromosome preparations were from human peripheral blood lymphocytes stimulated with PHA. Immunocytochemical detection of the hybridized probe was achieved using avidin-FITC and anti-avidin repetitive amplification steps. The slides were mounted in antifade medium containing 4,6-diamino-2-phenylindole DAPI-II for counterstaining of the chromosomes. Chromosomes were evaluated under a Zeiss fluorescence microscope equipped with appropriate filters for the visualization of FITC and DAPI. Arrows point to NES1-specific hybridization signals in the telomeric region of the long arm of both chromosomes 19 at band q13.3.

Fig. 4 A. Northern blot analysis of NES1 mRNA expression in prostate-derived normal, immortalized and tumor epithelial cells. Nylon membranes with 10 μg of total RNA from normal prostate tissue, immortalized prostate cell lines SV40-I and HPV18-I, and prostate tumor cell lines DU-145, LNCaP, ND1 and PC3, were hybridized with a ^{32}P-labelled NES1 probe and visualized by autoradiography. 76N normal mammary epithelial cell strain was used as a positive control. 36B4 probe was used as a loading control.

Fig. 4 B. Western blot analysis of NES1 protein in prostate-derived immortalized and tumor epithelial cell lines. Aliquots of culture supernatants derived from different cells (as indicated above) containing 300 μg protein were resolved by a SDS-10 % PAGE and transferred to PVDF membrane. Membranes were immunoblotted either with an anti-NES1 antiserum (above panel) or with an anti-PSA monoclonal antibody followed by goat anti-rabbit IgG or goat anti-mouse IgG horse-radish peroxidase conjugates, respectively. Detection was by enhanced chemiluminescence. Positive (+ve) control, supernatant from NES1-transfected MDA-MB-231 cells.

Figure 1 (A and B)

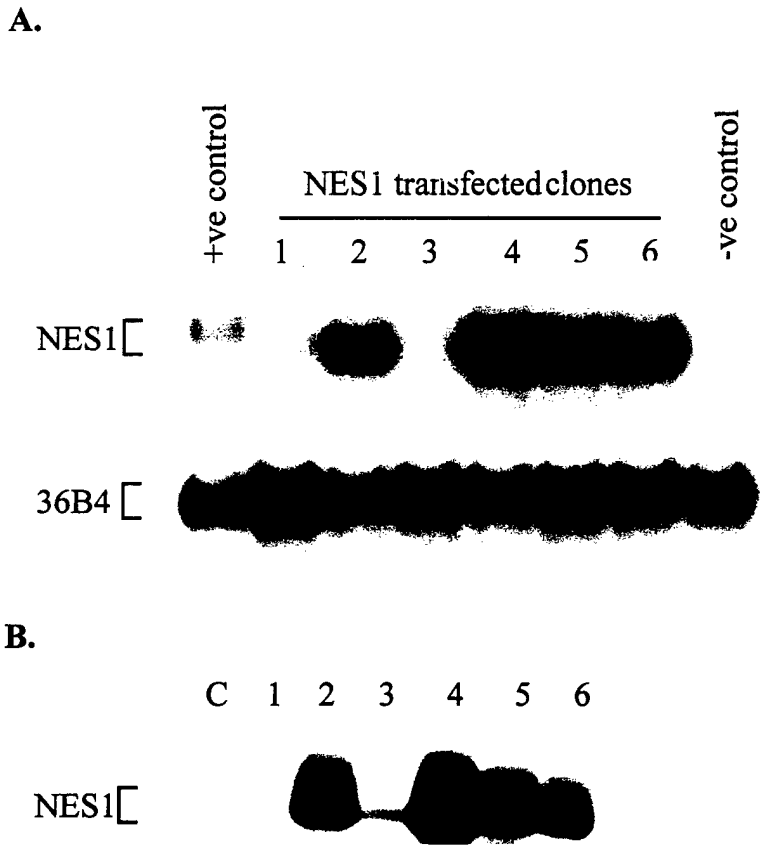

Figure 2A

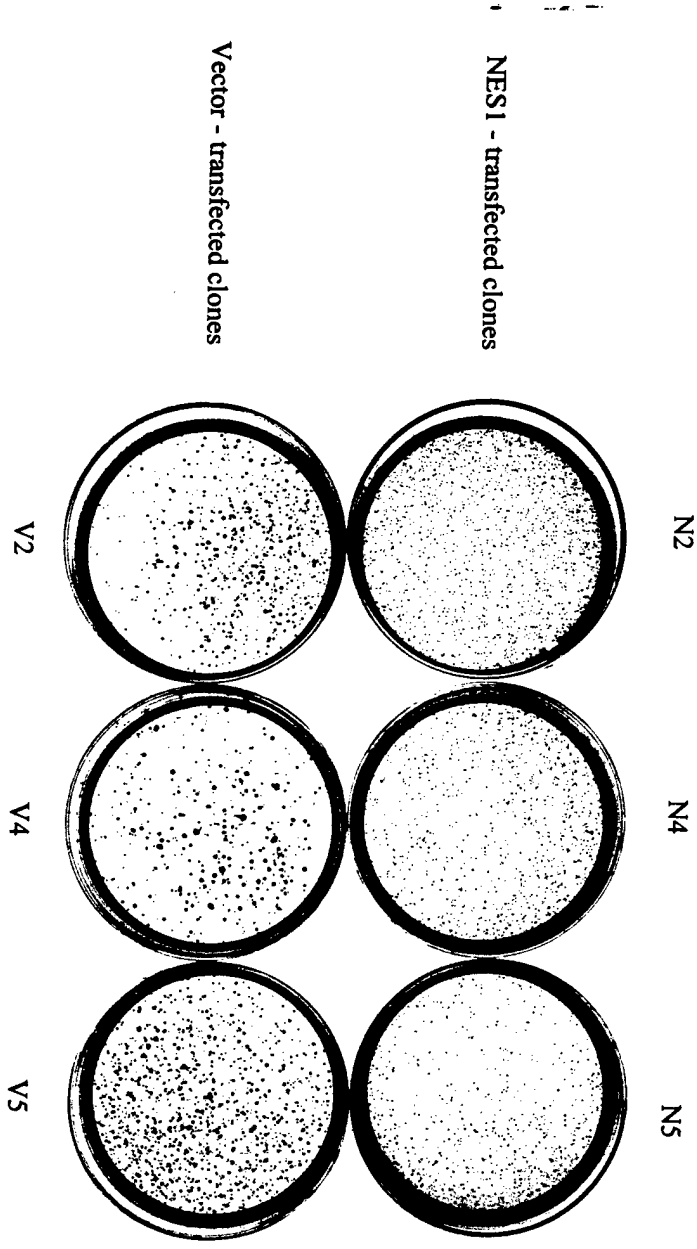
NES1 - transfected clones
Vector - transfected clones
N2
N4
N5
V2
V4
V5

Figure 2B

NES1 - transfected cells

Vector - transfected cells

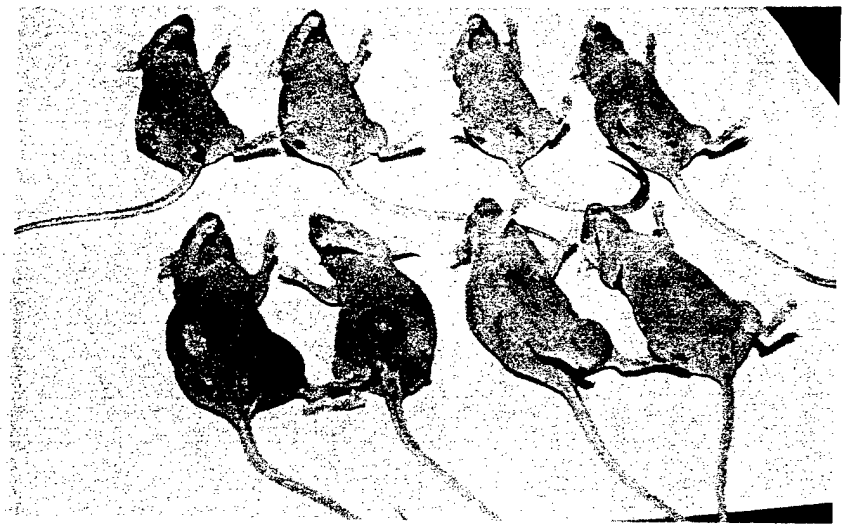

Figure 3

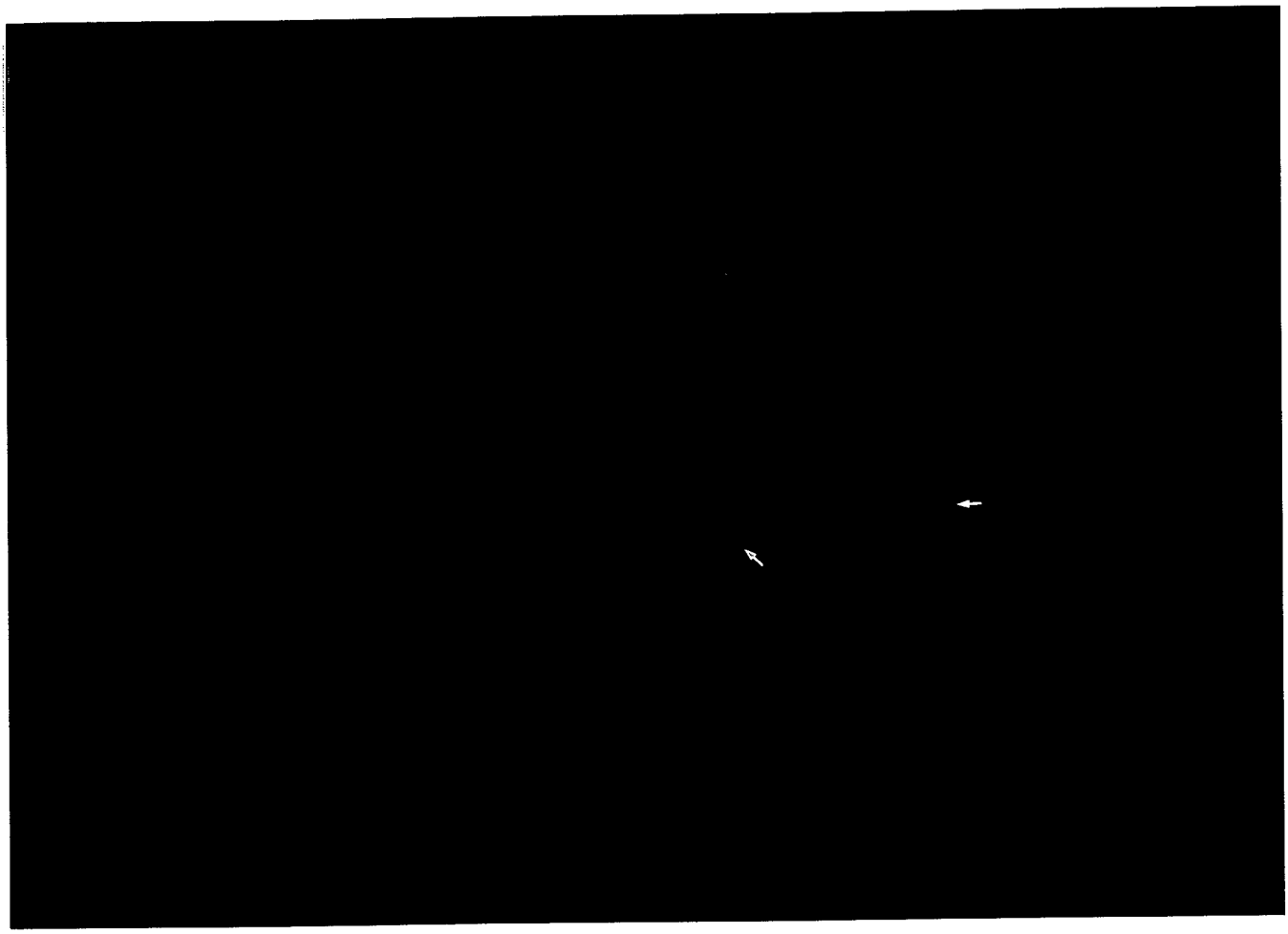

Figure 4A

A.

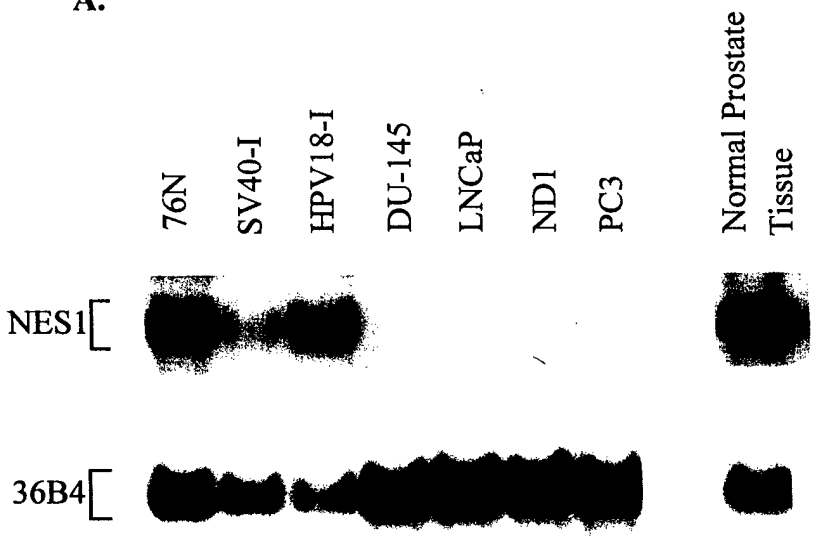
76N
SV40-I
HPV18-I
DU-145
LNCaP
ND1
PC3
Normal Prostate Tissue
NES1
36B4

Figure 4B